Title

Mutations in the P-type cation-transporter ATPase 4, PfATP4, mediate resistance to both aminopyrazole and spiroindolone antimalarials.

Journal

ACS chemical biology, 10(2)

ISSN

1554-8929

Authors

Flannery, Erika L
McNamara, Case W
Kim, Sang Wan
et al.

Publication Date

2015-02-01

DOI

10.1021/cb500616x

Peer reviewed

ACS chemical biology

Articles

pubs.acs.org/acschemicalbiology

Mutations in the P-Type Cation-Transporter ATPase 4, PfATP4, Mediate Resistance to Both Aminopyrazole and Spiroindolone Antimalarials

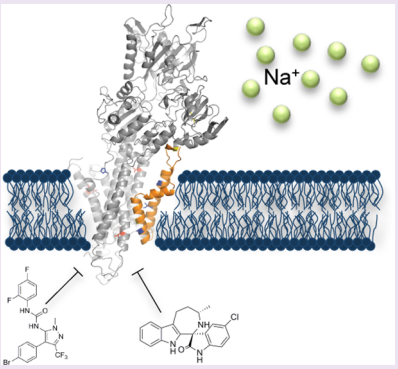
Erika L. Flannery,[†] Case W. McNamara,[§] Sang Wan Kim,[†] Tomoyo Sakata Kato,[§] Fengwu Li,[‡] Christine H. Teng,[†] Kerstin Gagaring,[§] Micah J. Manary,[†] Rachel Barboa,[§] Stephan Meister,[†] Kelli Kuhen,[§] Joseph M. Vinetz,[‡] Arnab K. Chatterjee,[§] and Elizabeth A. Winzeler*,[†]

[†]Division of Pharmacology and Drug Discovery, Department of Pediatrics, [‡]Division of Infectious Diseases, Department of Medicine, University of California San Diego School of Medicine, La Jolla, California 92093, United States

[§]The Genomics Institute of the Novartis Research Foundation, San Diego, California 92121, United States

Supporting Information

ABSTRACT: Aminopyrazoles are a new class of antimalarial compounds identified in a cellular antiparasitic screen with potent activity against *Plasmodium falciparum* asexual and sexual stage parasites. To investigate their unknown mechanism of action and thus identify their target, we cultured parasites in the presence of a representative member of the aminopyrazole series, GNF-Pf4492, to select for resistance. Whole genome sequencing of three resistant lines showed that each had acquired independent mutations in a P-type cation-transporter ATPase, PfATP4 (PF3D7_1211900), a protein implicated as the novel *Plasmodium* spp. target of another, structurally unrelated, class of antimalarials called the spiroindolones and characterized as an important sodium transporter of the cell. Similarly to the spiroindolones, GNF-Pf4492 blocks parasite transmission to mosquitoes and disrupts intracellular sodium homeostasis. Our data demonstrate that PfATP4 plays a critical role in cellular processes, can be inhibited by two distinct antimalarial pharmacophores, and supports the recent observations that PfATP4 is a critical antimalarial target.



The need to identify new drugs to combat malaria has resulted in high-throughput cellular screening campaigns that have revealed thousands of small molecule inhibitors with antimalarial activity;[1−3] several of which are currently in clinical trials.[4−8] Although a successful method of drug discovery,[9,10] a remaining challenge with cellular screening is in identifying the targets of lead compounds. Target identification is not essential for drug development, but nevertheless improves medicinal chemistry efforts and allows for the design of target-based high-throughput assays that may yield additional potent inhibitors of a target of interest.

The current number of chemically validated targets in *Plasmodium* spp. remains small[11,12] and includes the cytochrome bc_1 complex,[13] inhibition of hemozoin formation,[14] phosphatidylinositol-4-OH kinase,[6] dihydroorotate dehydrogenase,[15] dihydrofolate reductase,[16] and the P-type ATPase, PfATP4.[5] As with other antimalarial targets, P-type ATPases are important druggable targets in humans with several clinically relevant inhibitors.[17−19] Specifically, PfATP4 belongs to a subfamily of these proteins (family IID) that function to extrude monovalent cations (sodium, lithium, and potassium) from inside the cell. Their presence is limited to lower eukaryotes (fungi, protozoan, and bryophytes) making them an attractive drug target.[20,21] PfATP4 is the target of the spiroindolones, a novel antimalarial chemical class discovered in a cellular screen.[5,22,23] In a Phase II clinical trial, this class was shown to induce faster parasite clearance times than the current standard of care, artemisinin, and was shown to be active against artemisinin resistant parasites.[24]

In this study, we use a chemical genomics approach[25] to identify PfATP4 as the target of another novel class of inhibitors with antimalarial activity, the aminopyrazoles, which are structurally distinct from the spiroindolones. We further show that the phenotypes of spiroindolone- and aminopyrazole-treated parasites are similar. We additionally identify a third chemotype that interacts with PfATP4. Convergence on this target by multiple chemophores highlights the critical function of this protein in the parasite. Overall, these data suggest that the number of druggable targets in *P. falciparum* may be smaller than first hypothesized.

RESULTS AND DISCUSSION

***In Vitro* Evolution in the Presence of GNF-Pf4492 Identifies Resistance-Conferring Mutations in *P. falciparum pfatp4*.** Aminopyrazoles were identified in a high-throughput cellular screen against *P. falciparum* asexual blood

Received: August 1, 2014
Accepted: October 16, 2014
Published: October 16, 2014

stages.[1] A representative compound from the aminopyrazole series, GNF-Pf4492 (Figure 1a) (N-[4-(4-bromophenyl)-1-

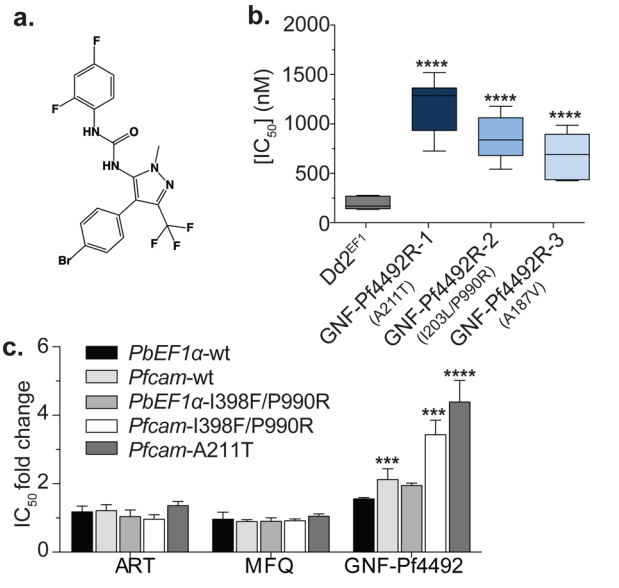


Figure 1. Evolution of *P. falciparum* GNF-Pf4492-resistant parasite lines. (a) Chemical structure of GNF-Pf4492, a representative compound from the aminopyrazole series. (b) *In vitro* drug sensitivities of the clonal GNF-Pf4492 evolved resistant lines and the $Dd2^{EF1}$ parent to GNF-Pf4492 were determined using a SYBR Green I-based cell proliferation assay. Bars represent mean IC_{50} from a minimum of 3 experiments conducted in duplicate. Error bars = 95% confidence interval. (c) IC_{50} fold change in transgenic parasite lines harboring additional wild-type *pfatp4* copies (*PbEF1α*-wt and *Pfcam*-wt) or mutated *pfatp4* (*PbEF1α*-I398F/P990R, *Pfcam*-I398F/P990R or *Pfcam*-A211T) under the control of the *P. berghei EF1α* promoter or the stronger *P. falciparum camodulin* promoter compared to $Dd2^{attB}$ (contains only the isogenic recombination site). Significance values were determined using one-way ANOVA followed by Dunnett's multiple comparison post-test to test for a difference in mean log(IC_{50}) value between each strain and the parent: ****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$.

methyl-3-(trifluoromethyl)-1H-pyrazol-5-yl]-N′-(2,4-difluorophenyl)urea, has a mean half maximal (50%) inhibitory concentration (IC_{50}) of 184.1 nM (95% confidence interval (CI) 141.3−239.9) against asexual stages of the multidrug resistant *P. falciparum* strain, Dd2, and demonstrates no cytotoxicity against the human heptoma cell line Huh7 (>30 μM). Given that the aminopyrazoles have chemical structures that are distinct from currently recommended antimalarials and scaffolds in development, we sought to identify the target of the aminopyrazoles by evolving resistant parasites and examining their genomes for changes that contribute to the resistance phenotype.[25]

GNF-Pf4492 drug-resistant lines (GNF-Pf4492R) were selected for in three independent cultures of the multidrug resistant, clonal *P. falciparum* Dd2 strain ($Dd2^{EF1}$) by exposure to sublethal concentrations of GNF-Pf4492 for 70 days (SI Figure S1). Each of the resistant clones exhibited mean IC_{50} values that were significantly greater than the $Dd2^{EF1}$ parental line (Figure 1b, SI Table S1) ($p < 0.0001$) and were 1170 nM (95% CI 920.5−1483), 811.0 nM (631.0−1040), and 631.0 nM (458.1−867.0) for GNF-Pf4492R-1, −2 and −3, respectively. None of the mutants appeared to endure any fitness cost as they grew at the same rate as the $Dd2^{EF1}$ parent. GNF-Pf4492 resistance was stable in these lines; that is, when grown without selective pressure for several months GNF-Pf4492 resistance was still observed. To identify genetic changes that contribute to the GNF-Pf4492 resistance phenotype we compared the genomic sequence of each resistant clone with the sequence of the $Dd2^{EF1}$ parent (Table 1).

Single-nucleotide variants (SNVs), as well as copy number variants (CNVs), contribute to drug resistance in *Plasmodium spp.*; therefore, we looked for both types of genetic changes in our resistant lines.[5,26,27] We did not observe any unique CNVs in the GNF-Pf4492R lines compared to the $Dd2^{EF1}$ parent (Figure S2) but did identify SNVs that were unique to the resistant lines, and therefore arose during selective pressure with GNF-Pf4492. A total of 15 868, 15 983, and 16 367 SNVs were confidently identified in GNF-Pf4492R-1, GNF-Pf4492R-2, and GNF-Pf4492R-3, respectively, compared to the 3D7 reference genome (Table 1). After comparative analysis with the $Dd2^{EF1}$ parental line, 74 SNVs were found to be unique: 28 in GNF-Pf4492R-1, 22 in GNF-Pf4492R-2, and 24 in GNF-Pf4492R-3 (Table 1 and SI Table S2). Of these, six resulted in a nonsynonymous amino acid substitution in an exon of an open reading frame (two in each line) (Table 1) and remarkably, all three lines contained SNVs in PF3D7_1211900 (formerly

Table 1. Whole Genome Sequencing Identifies SNVs in *pfatp4* in All GNF-Pf4492 Resistant Lines

	GNF-Pf4492 resistant lines		
	GNF-Pf4492R-1	GNF-Pf4492R-2	GNF-Pf4492R-3
genome coverage (*x*)	41	55	55
% covered by 15 or more reads	80.3	78.1	86.5
SNVs identified			
raw[a]	63 206	62 951	67 348
quality[b]	15 868	15 983	16 367
unique[c]	28	22	24
intergenic	21	16	20
intronic	4	4	2
synonymous	0	0	0
nonsynonymous	2	2	2
gene (mutation)	PF10_0182: conserved protein (K309N), PFL0590c: PfATP4 (A211T)	PFL0590c: PfATP4 (I203L), PFL0590c: PfATP4 (P990R)	PF10_0366: ADP/ATP transporter (I301N), PFL0590c: PfATP4 (A187V)

[a]After alignment to *P. falciparum* 3D7 reference genome. [b]Quality filters based on parameters defined in Methods. [c]Compared to Dd2 parent. No., number; SNVs, single-nucleotide polymorphisms; PfATP4, P-type cation ATPase 4.

dx.doi.org/10.1021/cb500616x | ACS Chem. Biol. 2015, 10, 413−420

PFL0590c), the gene that encodes the P-type cation-transporting ATPase, PfATP4. GNF-Pf4492R-1 and GNF-Pf4492R-3 each harbored one mutation in *pfatp4* (Ala211Thr and Ala187Val, respectively) while GNF-4492R-2 bore two mutations (Ile203Leu and Pro990Arg) (Table 1). These two mutations were the only nonsynonymous substitutions identified in the entire GNF-Pf4492R-2 genome. Mutations in all resistant clones were covered by 76 to 93 reads (SI Table S2), lending high confidence to the base calls, and were further confirmed by Sanger sequencing.

In addition to the mutations identified in *pfatp4*, two additional candidate nonsynonymous coding SNVs were identified in GNF-Pf4492R-1 and -3. A nonsynonymous mutation in PF3D7_1018900 (formerly PF10_0182), a gene encoding a conserved protein of unknown function, was identified in GNF-Pf4492R-1. Sanger sequencing showed the expected parental sequence of PF3D7_1018900 in GNF-Pf4492R-1. It was noted that this SNV occurs in a homopolymer tract, which causes difficulty for the read alignment program, therefore causing an isolated false positive call (SI Figure S3a). In GNF-Pf4492R-3, a third nonsynonymous coding SNV (encoding the mutation Ile301Asn) was identified in the last amino acid of the polypeptide encoded by PF3D7_1037300 (formerly PF10_0366). This gene encodes an ADP/ATP transporter, a protein that enables ATP and ADP to traverse the inner mitochondrial membrane.[28] The human ADP/ATP transporter, which is critical for cellular respiration, can be chemically inhibited and is the target of the poisons, bongkrekic acid and atractyloside.[29] Interestingly, we observed a K544N mutation in *pfcdpk5* (Pf3D7_1337800) in all three resistant lines (SI Figure S3b). Although this SNV is not present in the published Dd2 sequence, it was found in our $Dd2^{EF1}$ line. We thus believe that this SNV was not acquired during GNF-Pf4492 selective pressure.

Because resistance to aminopyrazoles could be conferred by mutations in *pfatp4*, one of the noncoding genetic changes, the mutation in the ADP/ATP transporter, or a yet unidentified locus, we evaluated a transgenic *P. falciparum* Dd2 line containing an *attB* ($Dd2^{attB}$) site integrated at the *cg6* locus[30] expressing one extra gene copy of wild-type *pfatp4* under the control of the *P. berghei* elongation factor-1 α (*EF1α*) promoter[5] or the stronger, *P. falciparum (camodulin) cam* promoter.[5,30] Both stable overexpression transgenic lines showed $a > 1.5\times$ shift in resistance to GNF-Pf4492 compared to the $Dd2^{attB}$ parent (Figure 1c, SI Table S1). We observed no change in the IC_{50} of control compounds and Western blot analysis with antibodies to PfATP4 confirmed low levels of overexpression (SI Figure S4a) relative to the $Dd2^{attB}$ parent. Because resistance in the GNF-Pf4492R-lines was caused by mutant alleles and not overexpression (SI Figure S4b), we also evaluated $Dd2^{attB}$ strains expressing mutated copies of *pfatp4*. The *Pfcam*-A211T strain harbors the mutated *pfatp4* observed in the GNF-Pf4492R-1 line. Two additional pre-existing transgenic lines bearing *pfatp4* with two mutations (Ile398Phe and Pro990Arg) under control of the *P. berghei EF1α* promoter or the *P. falciparum cam* promotor were also evaluated for cross-resistance.[5] We saw a 2-fold increase in resistance in the *PbEF1α*-I398F/P990R line. Significantly, we observed a 3.6 and 4.4 fold shift in resistance in the *Pfcam*-I398F/P990R line and *Pfcam*-A211T line respectively, but no shift for other antimalarials. This increase in resistance in the mutant *pfatp4* expressing strains is likely smaller than expected due to concurrent expression of both wild-type and mutant PfATP4. Western Blot analysis showed that this does not appear to be due only to overexpression of the protein (SI Figure S4a). These data show that mutations in *pfatp4* alone confer resistance to aminopyrazoles.

Aminopyrazole-Resistant Parasite Lines Exhibit Cross-Resistance with the Spiroindolone Drug Series. Mutations in *pfatp4*, whose protein product is implicated in parasite sodium tolerance,[21] also confer resistance to spiroindolones, a novel class of antimalarial that are currently in clinical trials.[5,23] The *pfatp4* mutations associated with spiroindolone resistance (SI Table S3) were previously identified using the same methodology, yet with small molecule inhibitors from the spiroindolone class, either KAE609 (formerly NITD609) or KAE678 (formerly NITD678) (Figure 2a,b).[5] The *pfatp4* SNVs detected in all aminopyrazole- and spiroindolone resistant lines are present at or near the predicted transmembrane (TM) domain of the protein, where cations are translocated, suggesting a shared structure−function relationship (Figure 2c). All GNF-Pf4492-associated mutations, except Pro990Arg, occurred in TM helices 1 and 2 (model backbone colored orange) and corresponded at or near reported KAE678 resistance-conferring mutations: Ala184Ser (yellow), Ile203Met (yellow; identical location of GNF-Pf4492 mutation), and Gly223Arg (yellow) (Figure 2c). The Pro990Arg mutation is located 5 residues before the predicted start of TM helix 7 and, thus, resides near the TM region. Curiously, as does GNF-Pf4492R-3, KAE678R-2 also possessed a nonsynonymous mutation in the ADP/ATP antiporter (Ile119Ser), suggesting that this may function as a compensatory mutation or possibly an additional target.[28]

Because the spiroindolone chemotype is structurally different from the aminopyrazole class, we did not expect resistance to both chemical classes would be conferred by mutations in the same gene. We characterized the extent of cross-resistance using three closely related members of the spiroindolone series, KAE609, KAE678, and KAF246 (formerly NITD246[21]) and found GNF-Pf4492R-2 and -3 were 3- to 8-fold more resistant, to the spiroindolones than the $Dd2^{EF1}$ parent (Figure 2d, SI Table S1). Surprisingly, GNF-Pf4492R-1, the line most resistant to the aminopyrazole, showed an increased sensitivity to the spiroindolones (Figure 2d). This line was 7- to 20-fold more sensitive to all of the spiroindolone compounds tested compared to the $Dd2^{EF1}$ parent. To further investigate this curious observation, we tested the *Pfcam*-A211T transgenic line for cross resistance with KAF246. The *Pfcam*-A211T showed some signs of sensitivity while the *Pfcam* I398F/P990R was >2-fold more resistant to KAF246 (Figure 2e). Failure to see more sensitivity introduced with the *Pfcam*-211T mutation is again most likely due to the presence of both wild-type and mutant PfATP4. Similarly, the six independently evolved spiroindolone-resistant lines were also resistant to GNF-Pf4492, exhibiting 3−15 fold higher IC_{50} values compared to the parent (Figure 2f, SI Table S1). No significant change was observed in IC_{50} for the controls artemisinin and mefloquine, demonstrating this effect is specific and not due to a multidrug efflux mechanism.

To determine if any other publicly available compounds would have activity against PfATP4, we screened several inhibitors from the "malaria box," a set of compounds previously identified in cellular screens with activity against blood-stage *P. falciparum*, for cross-resistance with the GNF-Pf4492R lines.[31] Two of these compounds, MMV666124 (Figure 3a) and MMV020660 (Figure 3b), showed cross-

dx.doi.org/10.1021/cb500616x | ACS Chem. Biol. 2015, 10, 413−420

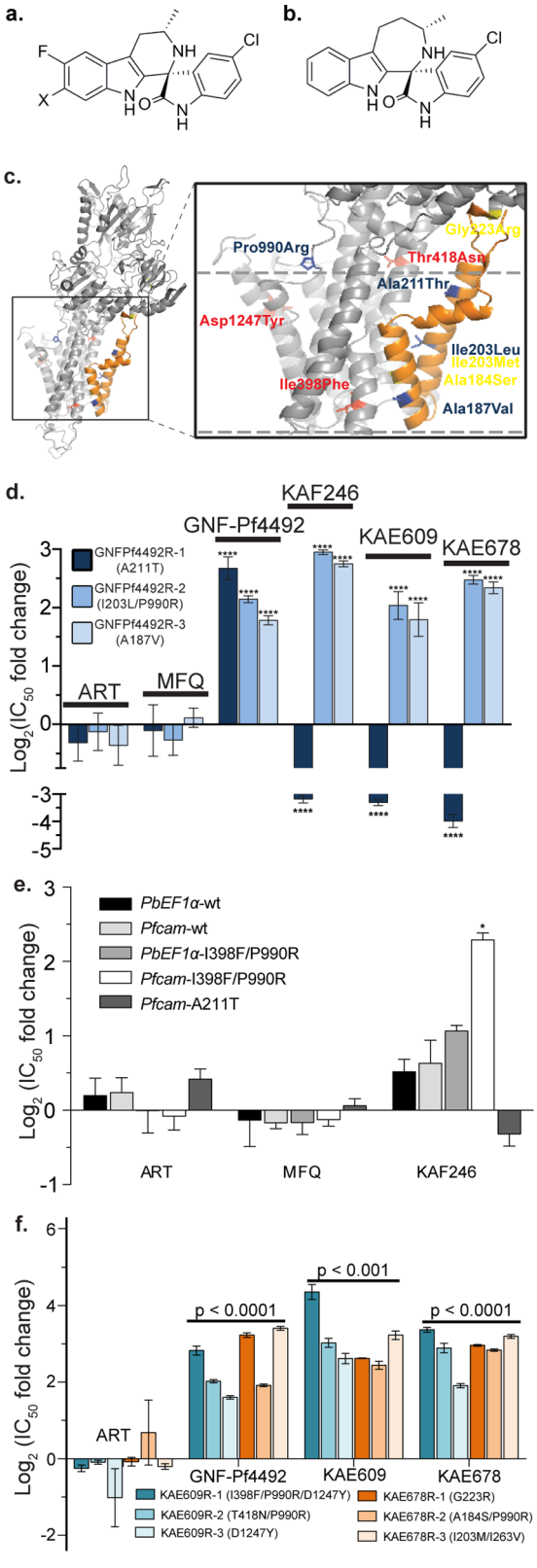


Figure 2. Mutations in *pfatp4* confer cross-resistance between GNF-Pf4492 and the spiroindolone class. Chemical structures of representative spiroindolones (a) KAE609 when X = Cl and KAF246 when X = F. (b) KAE678 is distinguished by a 7-membered ring in the tricyclic system. (c) A PfATP4 homology model shows the location of resistance-conferring mutations specific to the aminopyrazole GNF-Pf4492 (blue) and the spiroindolones KAE609 (red) and KAE678 (yellow). Nearly all resistance-conferring mutations

Figure 2. continued

occur within or near PfATP4 transmembrane domains (approximated by dashed lines). Transmembranes 1 and 2 are in orange. (d) The three GNF-Pf4492-resistant lines—GNF-Pf4492R-1, GNF-Pf4492R-2, and GNF-Pf4492R-3—were tested for cross-resistance against a panel of spiroindolones (KAE609, KAE678, and KAF246). The IC_{50} shift is relative to the GNF-Pf4492-sensitive $Dd2^{EF1}$. Artemisinin (ART) and mefloquine (MFQ) were used as controls. (e) IC_{50} $\log_2$ fold change in transgenic lines harboring either wild-type *pfatp4* (*PbEF1α*-wt and *Pfcam*-wt) or mutated *pfatp4* (*PbEF1α*-I398F/P990R, *Pfcam*-I398F/P990R or *Pfcam*-A211T). (f) Resistant lines (three each) were independently evolved to spiroindolone analogs KAE609 and KAE678. These lines were tested for cross-resistance against GNF-Pf4492. ART and MFQ were used as controls. Significance values were determined using one-way ANOVA followed by Dunnett's multiple comparison post-test to test for a difference in mean $\log(IC_{50})$ value between each strain and the parent: ****$p < 0.0001$; *$p < 0.01$.

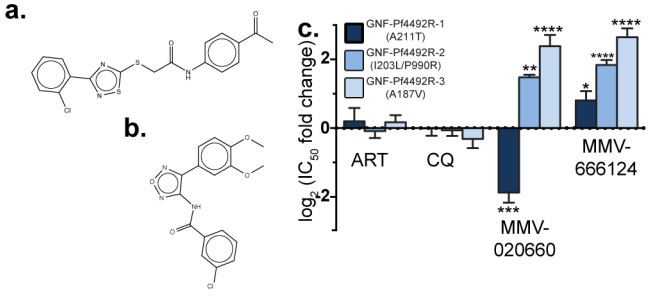


Figure 3. GNF-Pf4492-R lines show cross-resistance to the malaria box inhibitors MMV666124 and MMV020660. Chemical structure of compounds known to have blood-stage antimalarial activity (a) MMV666124 and (b) MMV020660. (c) Log_2 fold change in IC_{50} for GNF-Pf4492R lines compared to the $Dd2^{EF1}$ parent. Bars represent mean $\log_2(IC_{50}$ fold change) from a minimum of 3 experiments conducted in duplicate. Error bars = SEM; Significance values were determined using one-way ANOVA followed by Dunnett's multiple comparison post-test to test for a difference in mean $\log_2(IC_{50})$ between each strain and the $Dd2^{EF1}$ parent; *$p < 0.05$, **$p < 0.01$.

resistance with the GNF-Pf4492R lines (Figure 3c). As with the phenotype observed with the spiroindolones, GNF-Pf4492R-1 did not exhibit cross-resistance with MMV020660 but rather was more sensitive than the $Dd2^{EF1}$ parent. Interestingly, the structure of MMV020660 shows some similarity to GNF-Pf4492 (Figure 3b).

Aminopyrazoles and Spiroindolones Induce Similar Phenotypes *In Vitro*. Given that mutated PfATP4 mediates aminopyrazole resistance, we hypothesized that the aminopyrazoles and spiroindolones share a mechanism of action. Therefore, we sought to determine whether parasites treated with inhibitors from either class produced the same phenotype. Synchronized cultures were treated with 10× IC_{50} GNF-Pf4492 or KAF246 to observe when the compounds act during the parasite life cycle. Neither culture treated with inhibitor advanced past the early trophozoite stage, arresting in the ring or early trophozoite stages (Figure 4a). Furthermore, similar to the spiroindolones, GNF-Pf4492 diminished protein synthesis activity in the trophozoite stage of the Dd2 parent at 10× and 100× IC_{50} concentrations as measured by incorporation of radiolabeled methionine and cysteine (SI Figure S5a). Conversely, protein synthesis progressed unabated in the KAE609R-1 clone after addition of either 100× KAF246 or GNF-Pf4492 (SI Figure S5b).

dx.doi.org/10.1021/cb500616x | *ACS Chem. Biol.* 2015, 10, 413−420

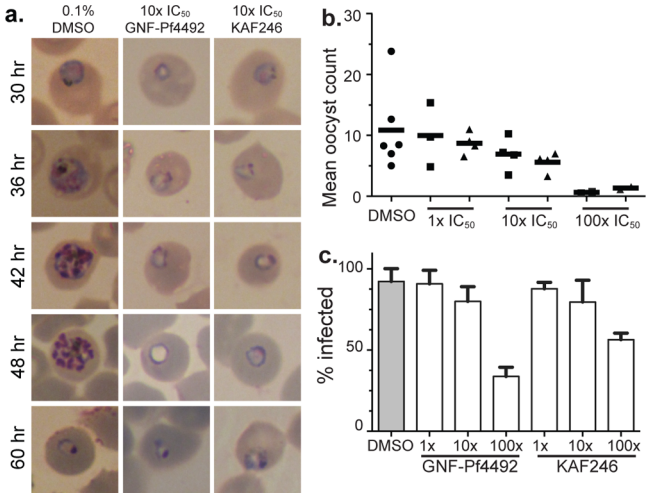


Figure 4. Phenotypic characterizations of parasites treated with aminopyrazoles or spiroindolones. (a) The lifecycle stage of action of GNF-Pf4492 and KAF246 were determined by addition of either 10× IC_{50} of GNF-Pf4492 or KAF246 and observation over a 60 h period. (b, c) Standard membrane feeding with *P. falciparum* to determine inhibitor action on transmission. Ten to forty mosquitoes per feeding were dissected. (b) Geometric mean number of oocysts counted per feeding. Bars represent median. Squares, GNF-Pf4492 treated; triangles, KAF246 treated. (c) The percent of mosquitoes that had one or more oocysts per the total number of mosquitoes dissected.

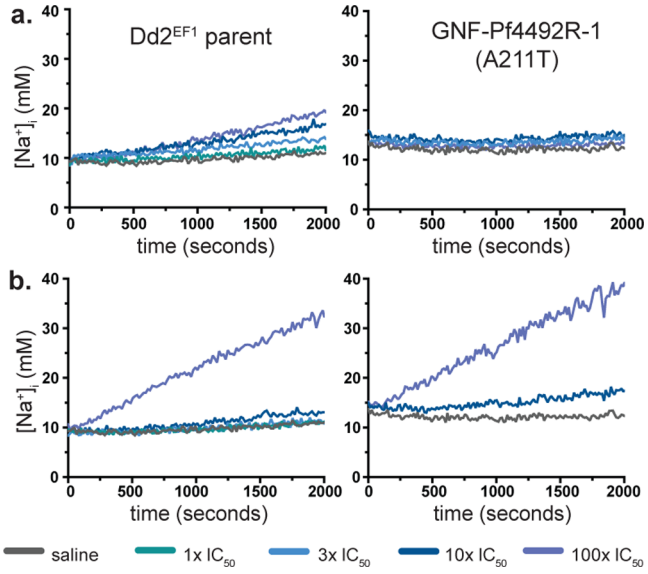


Figure 5. Structure–function relationship between PfATP4 mutations and compound treatment. Effects of (a) aminopyrazole and (b) spiroindolone inhibitors on intracellular sodium concentration $[Na^+]_i$ in the $Dd2^{EF1}$ and GNF-Pf4492R-1 strains. $[Na^+]_i$ traces for extracted trophozoite-stage parasites treated with saline or inhibitor immediately prior to fluorescence acquisition. Inhibitors were added at 1 × , 3×, 10×, or 100× the IC_{50} for the $Dd2^{EF1}$ parent. Traces are representative of those obtained from a minimum of four independent experiments performed in duplicate. Calibration curves were generated for each strain and each experimental replicate to determine $[Na^+]_i$.

While phenocopy was observed for drug-treated parasites in the asexual blood stages, we continued characterization of these inhibitors in the liver and sexual blood stages. Addition of GNF-Pf4492 or KAF246 to *P. yoelii* sporozoite-invaded HepG2 cells[4] resulted in no detectable reduction in parasite growth at 10 μM, the highest concentration tested (SI Table S4). Van Pelt Koops et al. demonstrated that the spiroindolones are capable of blocking transmission to mosquitoes.[32] Therefore, we used a standard membrane-feeding assay to assess the effectiveness of GNF-Pf4492 in preventing *P. falciparum* oocyst development in the mosquito[33] GNF-Pf4492 or KAF246 inhibitors were added at three different concentrations directly to a parasite blood meal. This resulted in a dose-dependent decrease in the number of oocysts formed in the mosquito midguts 8 days after feeding (Figure 4b). The strongest effect was observed for the 100× IC_{50} concentration for both GNF-Pf4492 and KAF246 (mean oocyst count of 0.63 and 1.33, respectively). At these concentrations, 30 nM KAF246 and 15 μM GNF-Pf4492, a respective reduction of 60% and 38% of the control was observed. Additionally, as compound concentration was increased, the percentage of mosquitoes infected also decreased (Figure 4c).

GNF-Pf4492 Causes an Increase in Intracellular Sodium Concentration in *P. falciparum*. Recent studies show that PfATP4 functions to transport sodium against a concentration gradient, out of the cell, maintaining a low intracellular sodium concentration in the presence of the high sodium environment of the infected red blood cell.[21,34,35] Therefore, we sought to determine whether both compounds would affect sodium concentration within the parasite cytoplasm. When 100× IC_{50} GNF-Pf4492 (Figure 5a) or KAF246 (Figure 5b) was added, a rapid, steady, and comparable increase in intracellular sodium concentration ($[Na^+]_i$) was observed over a 30 min period and this response was demonstrated to be dose-dependent in the presence of GNF-Pf4492. For GNF-Pf4492R-1 and -2, when treated with compound these lines maintained the same $[Na^+]_i$ over the observation period (Figure 5a, SI Figure S6a). While a dose-dependent response was observed for GNF-Pf4492R-3, $[Na^+]_i$ concentrations did not reach those of the $Dd2^{EF1}$ after 30 min of observation after addition of the inhibitor (SI Figure S6a). In the presence of KAF246, in the GNF-Pf4492R-1 mutant, which is hyper-sensitive to KAF246, a similar pattern of increase in $[Na^+]_i$ was observed as the parent (Figure 5b). For the highest concentration of KAF246 used (100× IC_{50}), increases in $[Na^+]_i$ were similar for the GNF-Pf4492R-2 and -3 mutants, although the final $[Na^+]_i$ remained suppressed below that observed in the parental line (SI Figure S6b). The resting $[Na^+]_i$ concentrations for GNF-Pf4492R-1 and -2 were consistently higher than that of the parent (SI Table S5).

Conclusions. New phenotypic screening efforts have unveiled novel antimalarial chemotypes with correspondingly novel mechanisms of action.[4,5,36] Inherently, the identification of targets of compound hits originating from cellular screens requires deconvolution. Target identification for one of these compounds, the aminopyrazole GNF-Pf4492,[1] reveals it phenocopies the spiroindolone series, a chemical class shown in Phase II clinical trials to induce faster parasite clearance times than artemisinin.[5,24] We demonstrate that cross-resistance between aminopyrazole and spiroindolone series is mediated by mutations in *pfatp4*, a gene encoding a P-type ATPase that functions as a Na^+/H^+ pump at the parasite plasma membrane.[21] A third chemotype associated with resistance-conferring mutations in *pfatp4*, dihydroisoquinolones, (http://www.mmv.org/research-development/rd-portfolio),[37] has progressed to preclinical evaluation as well. The convergence on the same target by inhibitors of unrelated compound classes is not uncommon for antimalarials and does not preclude their

dx.doi.org/10.1021/cb500616x | ACS Chem. Biol. 2015, 10, 413–420

importance as good drug targets. In the case of cytochrome *bc1*, the validated mitochondrial protein target of atovaquone,[38,39] although several other inhibitors target it,[27,40,41] atovaquone is used clinically with wide success for prophylaxis.[42] Identification of mutations in *pfatp4* that mediate resistance to multiple compound classes suggests its importance as a drug target, although the novel mechanism of action is unclear.

Insight into the mechanism of action of PfATP4 targeting inhibitors is gleaned from the location of amino acid changes conferring resistance. The *pfatp4* mutations we identify localize to the transmembrane domain suggesting inhibitors bind in the clefts within transmembrane helices. Clinically relevant p-type ATPase inhibitors bind to transmembrane helices leading to poor cation recognition or unfavorable enzymatic conformations.[18] We speculate that PfATP4 targeting compounds disrupt sodium export in an analogous manner to other P-type ATPase targeting drugs and that there is most likely a small structural element common to the two classes that accounts for both compound classes binding to the same druggable pocket. Resistance conferring PfATP4 mutations disrupt binding of the inhibitor to this pocket and such mutations could be expected to alter the function of the enzyme, which may be reflected in the slightly elevated resting sodium concentrations observed in two of the resistant lines. In the case of the mutations observed in the GNF-Pf4492R-1 line, a single mutation renders the strain resistant to the aminopyrazole yet sensitive to the spiroindolone (as phenomena observed with other P-type ATPase inhibitors[43]), suggesting the binding sites of each inhibitor are overlapping, yet unique.

Although our data suggests PfATP4 is the target of the spiroindolone and aminopyrazole compound series, alternative explanations exist, including a different biological function for PfATP4 (i.e., as a nonselective drug transporter), mutation of PfATP4 as a secondary, compensatory mutation in response to an as-of-yet unidentified target and last, PfATP4 function is sodium dependent, thus acting downstream of the true target. We evaluated the activity of other antimalarials (mefloquine and artemisinin) against aminopyrazole resistant lines and noted a low-incidence of cross-resistance, a finding inconsistent with the function of PfATP4 as a nonselective drug transporter. Furthermore, if PfATP4 is only involved in inhibitor efflux, we also might expect different chemical phenotypes, which we do not observe—aminopyrazoles and spiroindolones behave similarly throughout the lifecycle. The expression of these mutants alone was sufficient to confer aminopyrazole resistance, suggesting these are not compensatory mutations nor that PfATP4 is downstream of the true target and that PfATP4 plays a direct role in the mechanism of action of aminopyrazole antimalarials.

Other reports have used *in silico* modeling to predict that the pyrazole series might act by disrupting the protein−protein interaction between the *P. falciparum* myosin motor component myosin A and the myosin tail interacting protein (MTIP), a complex necessary for parasite reinvasion of red blood cells.[44] Despite showing *P. falciparum* blood-stage activity, docking studies were done *in silico* thus not directly validating the interaction between MTIP and aminopyrazoles. In addition, we show aminopyrazoles are active throughout the asexual life cycle and not just during cell invasion or gliding motility, which requires the MTIP-myosin A interaction.[45,46]

One open question is whether PfATP4 is the target of or a gene involved in resistance to both the aminopyrazoles and the spiroindolones. While the underlying mechanism by which PfATP4 inhibitors affect sodium levels in the cell remains to be elucidated, that mutations in PfATP4 are responsible for resistance to two (and possibly three) structurally unrelated compounds has important implications for future drug discovery efforts. It is difficult to imagine that convergence on PfATP4 is coincidental, given that both these drug series were identified by large chemical library screens and their relationship to PfATP4 identified by unbiased genomic sequencing of independently generated resistant lines. Because these screens produced antimalarial compounds acting through a variety of targets, we do not think the libraries are biased for PfATP4 inhibitors.[1−3] Therefore, there must be other reasons for target convergence. One possible explanation is that PfATP4 may represent a particularly strong drug target, perhaps owing to its molecular structure of physiologic importance. Although current Phase II clinical trials of the spiroindolones are very positive, they may have high production costs due to their chiral centers.[22] Investigation into other chemical classes, such as the aminopyrazoles, that interact with PfATP4 may represent an important alternative for combination therapies. Another possible reason for target convergence, may be that the number of suitable drug targets in *Plasmodium* is smaller than anticipated. Either way the role of PfATP4 as an important, novel antimalarial target warrants further study.

METHODS

See detailed version in SI Materials and Methods.

Evolution of Compound-Resistant Lines, Sensitivity Testing and Construction of Transgenic Parasite Lines. The *P. falciparum* multidrug resistant strain $Dd2^{EF1}$ was cultured in triplicate in the presence of increasing concentrations of GNF-Pf4492 to generate resistant mutants as previously described.[5] After ∼70 days of selection, parasites were cloned in 96-well plates by limiting dilution.[47] The half maximal (50%) inhibitory concentration (IC_{50}) of each compound against blood-stage *P. falciparum* $Dd2^{EF1}$ was determined in dose−response format using a SYBR Green I-based cell proliferation assay as previously described.[1] Liver-stage IC_{50} was determined as previously described.[4] Transgenic parasite lines were created as previously described.[5] Briefly, *P. falciparum* Dd2 containing the *attB* recombination site was transfected with the wild-type or mutant *pfatp4* transgene under the control of either the *P. berghei elongation factor-1α* 5′ UTR (*PbEF1α*) or the stronger *P. falciparum calmodulin* 5′ UTR (*Pbcam*).

Whole Genome Sequencing. DNA libraries of each sample were prepared using the Illumina TruSeq version 3 protocol (Illumina, Inc., San Diego, CA) of fragmentation, end-repair, and adapter ligation. Libraries were clustered and run on an Illumina HiSeq 2000 according to manufacturer's instructions. Fifty base pair, single-end reads were analyzed and aligned to the *P. falciparum* 3D7 reference genome (PlasmoDB v. 9.0) as previously described.[48] Sequence alignments were put through a custom quality control procedure.[48] SNVs were initially detected using the Genome Analysis Toolkit (GATK v1.6) and then filtered using the Plasmodium Type Uncovering Software, an integrated pipeline developed in our lab that can also call CNVs.[48] All nonsynonymous SNVs were confirmed using Sanger sequencing.

Phenotypic Assays for Parasite Characterization. An *E. coli* expression vector was constructed to express pure recombinant protein and mice were immunized to generate an antibody to the N-domain of PFATP4. Western blots were performed with 10 or 50 μg protein lysate and hybridized with the PfATP4 N-domain antibody to quantify protein levels in the cells. A previously generated homology model of PfATP4[5] was used to map the resistance-conferring mutations identified in the GNF-Pf4492-resistant lines. The PyMOL Molecular Graphics System (version 1.2r2, Schrödinger, LLC, Portland, OR) was used to render the model and prepare the figure. Compound stage of action and protein synthesis inhibition studies were conducted as previously described.[5] The standard membrane feeding assay was performed as previously described.[49] *P. falciparum*

dx.doi.org/10.1021/cb500616x | *ACS Chem. Biol.* 2015, 10, 413−420

NF54 was maintained *in vitro* in continuous cultivation with daily media changes and without the addition of fresh red blood cells to stimulate gametocytogenesis. Day 13, 15, and 17 gametocyte cultures that exhibited exflagellation were combined to form a mosquito bloodmeal supplemented with GNF-Pf4492 or KAF246 at 1×, 10×, or 100× the mean IC_{50} value for blood-stage parasites. Intracellular sodium concentration ($[Na^+]_i$) within the parasite was determined as previously described.[21] 50 μL of dye-loaded cells (2×10^9 cells/mL) were seeded into a black 384-well assay plate fluorescence was measured on an EnVision Multilabel reader (PerkinElmer, Waltham, MA).

■ ASSOCIATED CONTENT

Supporting Information

Detailed methods. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: ewinzeler@ucsd.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work partially supported by National Institutes of Health (NIH) (F32AI102567 to E.L. Flannery, 1R01AI0358-01A1 and 5R01AI090141-03 to E.A. Winzeler), the Bill and Melinda Gates Foundation (BMGF) (OPP1054480), and the Wellcome Trust (WT078285 and WT096157). We acknowledge the Medicines for Malaria Venture for providing the Malaria Box for screening and J. Walker for sequencing of the resistant parasite lines.

■ REFERENCES

(1) Plouffe, D., Brinker, A., McNamara, C., Henson, K., Kato, N., Kuhen, K., Nagle, A., Adrian, F., Matzen, J. T., Anderson, P., Nam, T. G., Gray, N. S., Chatterjee, A., Janes, J., Yan, S. F., Trager, R., Caldwell, J. S., Schultz, P. G., Zhou, Y., and Winzeler, E. A. (2008) *In silico* activity profiling reveals the mechanism of action of antimalarials discovered in a high-throughput screen. *Proc. Natl. Acad. Sci. U.S.A. 105*, 9059−9064.

(2) Gamo, F. J., Sanz, L. M., Vidal, J., de Cozar, C., Alvarez, E., Lavandera, J. L., Vanderwall, D. E., Green, D. V., Kumar, V., Hasan, S., Brown, J. R., Peishoff, C. E., Cardon, L. R., and Garcia-Bustos, J. F. (2010) Thousands of chemical starting points for antimalarial lead identification. *Nature 465*, 305−310.

(3) Guiguemde, W. A., Shelat, A. A., Bouck, D., Duffy, S., Crowther, G. J., Davis, P. H., Smithson, D. C., Connelly, M., Clark, J., Zhu, F., Jimenez-Diaz, M. B., Martinez, M. S., Wilson, E. B., Tripathi, A. K., Gut, J., Sharlow, E. R., Bathurst, I., El Mazouni, F., Fowble, J. W., Forquer, I., McGinley, P. L., Castro, S., Angulo-Barturen, I., Ferrer, S., Rosenthal, P. J., Derisi, J. L., Sullivan, D. J., Lazo, J. S., Roos, D. S., Riscoe, M. K., Phillips, M. A., Rathod, P. K., Van Voorhis, W. C., Avery, V. M., and Guy, R. K. (2010) Chemical genetics of *Plasmodium falciparum*. *Nature 465*, 311−315.

(4) Meister, S., Plouffe, D. M., Kuhen, K. L., Bonamy, G. M., Wu, T., Barnes, S. W., Bopp, S. E., Borboa, R., Bright, A. T., Che, J., Cohen, S., Dharia, N. V., Gagaring, K., Gettayacamin, M., Gordon, P., Groessl, T., Kato, N., Lee, M. C., McNamara, C. W., Fidock, D. A., Nagle, A., Nam, T. G., Richmond, W., Roland, J., Rottmann, M., Zhou, B., Froissard, P., Glynne, R. J., Mazier, D., Sattabongkot, J., Schultz, P. G., Tuntland, T., Walker, J. R., Zhou, Y., Chatterjee, A., Diagana, T. T., and Winzeler, E. A. (2011) Imaging of *Plasmodium* liver stages to drive next-generation antimalarial drug discovery. *Science 334*, 1372−1377.

(5) Rottmann, M., McNamara, C., Yeung, B. K., Lee, M. C., Zou, B., Russell, B., Seitz, P., Plouffe, D. M., Dharia, N. V., Tan, J., Cohen, S. B., Spencer, K. R., Gonzalez-Paez, G. E., Lakshminarayana, S. B., Goh, A., Suwanarusk, R., Jegla, T., Schmitt, E. K., Beck, H. P., Brun, R., Nosten, F., Renia, L., Dartois, V., Keller, T. H., Fidock, D. A., Winzeler, E. A., and Diagana, T. T. (2010) Spiroindolones, a potent compound class for the treatment of malaria. *Science 329*, 1175−1180.

(6) McNamara, C. W., Lee, M. C., Lim, C. S., Lim, S. H., Roland, J., Nagle, A., Simon, O., Yeung, B. K., Chatterjee, A. K., McCormack, S. L., Manary, M. J., Zeeman, A. M., Dechering, K. J., Kumar, T. R., Henrich, P. P., Gagaring, K., Ibanez, M., Kato, N., Kuhen, K. L., Fischli, C., Rottmann, M., Plouffe, D. M., Bursulaya, B., Meister, S., Rameh, L., Trappe, J., Haasen, D., Timmerman, M., Sauerwein, R. W., Suwanarusk, R., Russell, B., Renia, L., Nosten, F., Tully, D. C., Kocken, C. H., Glynne, R. J., Bodenreider, C., Fidock, D. A., Diagana, T. T., and Winzeler, E. A. (2013) Targeting *Plasmodium* PI(4)K to eliminate malaria. *Nature 504*, 248−253.

(7) Coteron, J. M., Marco, M., Esquivias, J., Deng, X., White, K. L., White, J., Koltun, M., El Mazouni, F., Kokkonda, S., Katneni, K., Bhamidipati, R., Shackleford, D. M., Angulo-Barturen, I., Ferrer, S. B., Jimenez-Diaz, M. B., Gamo, F. J., Goldsmith, E. J., Charman, W. N., Bathurst, I., Floyd, D., Matthews, D., Burrows, J. N., Rathod, P. K., Charman, S. A., and Phillips, M. A. (2011) Structure-guided lead optimization of triazolopyrimidine-ring substituents identifies potent *Plasmodium falciparum* dihydroorotate dehydrogenase inhibitors with clinical candidate potential. *J. Med. Chem. 54*, 5540−5561.

(8) Younis, Y., Douelle, F., Feng, T. S., Gonzalez Cabrera, D., Le Manach, C., Nchinda, A. T., Duffy, S., White, K. L., Shackleford, D. M., Morizzi, J., Mannila, J., Katneni, K., Bhamidipati, R., Zabiulla, K. M., Joseph, J. T., Bashyam, S., Waterson, D., Witty, M. J., Hardick, D., Wittlin, S., Avery, V., Charman, S. A., and Chibale, K. (2012) 3,5-Diaryl-2-aminopyridines as a novel class of orally active antimalarials demonstrating single dose cure in mice and clinical candidate potential. *J. Med. Chem. 55*, 3479−3487.

(9) Swinney, D. C., and Anthony, J. (2011) How were new medicines discovered? *Nat. Rev. Drug Discovery 10*, 507−519.

(10) Payne, D. J., Gwynn, M. N., Holmes, D. J., and Pompliano, D. L. (2007) Drugs for bad bugs: Confronting the challenges of antibacterial discovery. *Nat. Rev. Drug Discovery 6*, 29−40.

(11) Flannery, E. L., Chatterjee, A. K., and Winzeler, E. A. (2013) Antimalarial drug discovery—Approaches and progress towards new medicines. *Nat. Rev. Microbiol. 11*, 849−862.

(12) Fidock, D. A., Rosenthal, P. J., Croft, S. L., Brun, R., and Nwaka, S. (2004) Antimalarial drug discovery: Efficacy models for compound screening. *Nat. Rev. Drug Discovery 3*, 509−520.

(13) Mather, M. W., Darrouzet, E., Valkova-Valchanova, M., Cooley, J. W., McIntosh, M. T., Daldal, F., and Vaidya, A. B. (2005) Uncovering the molecular mode of action of the antimalarial drug atovaquone using a bacterial system. *J. Biol. Chem. 280*, 27458−27465.

(14) Pagola, S., Stephens, P. W., Bohle, D. S., Kosar, A. D., and Madsen, S. K. (2000) The structure of malaria pigment β-haematin. *Nature 404*, 307−310.

(15) Patel, V., Booker, M., Kramer, M., Ross, L., Celatka, C. A., Kennedy, L. M., Dvorin, J. D., Duraisingh, M. T., Sliz, P., Wirth, D. F., and Clardy, J. (2008) Identification and characterization of small molecule inhibitors of *Plasmodium falciparum* dihydroorotate dehydrogenase. *J. Biol. Chem. 283*, 35078−35085.

(16) Yuthavong, Y., Tarnchompoo, B., Vilaivan, T., Chitnumsub, P., Kamchonwongpaisan, S., Charman, S. A., McLennan, D. N., White, K. L., Vivas, L., Bongard, E., Thongphanchang, C., Taweechai, S., Vanichtanankul, J., Rattanajak, R., Arwon, U., Fantauzzi, P., Yuvaniyama, J., Charman, W. N., and Matthews, D. (2012) Malarial dihydrofolate reductase as a paradigm for drug development against a resistance-compromised target. *Proc. Natl. Acad. Sci. U.S.A. 109*, 16823−16828.

(17) Palmgren, M. G., and Nissen, P. (2011) P-type ATPases. *Annu. Rev. Biophys 40*, 243−266.

(18) Yatime, L., Buch-Pedersen, M. J., Musgaard, M., Morth, J. P., Lund Winther, A. M., Pedersen, B. P., Olesen, C., Andersen, J. P., Vilsen, B., Schiott, B., Palmgren, M. G., M?ller, J. V., Nissen, P., and

Fedosova, N. (2009) P-type ATPases as drug targets: Tools for medicine and science. *Biochim. Biophys. Acta 1787*, 207−220.
(19) Kuhlbrandt, W. (2004) Biology, structure, and mechanism of P-type ATPases. *Nat. Rev. Mol. Cell Biol. 5*, 282−295.
(20) Rodriguez-Navarro, A., and Benito, B. (2010) Sodium or potassium efflux ATPase a fungal, bryophyte, and protozoal ATPase. *Biochim. Biophys. Acta 1798*, 1841−1853.
(21) Spillman, N. J., Allen, R. J., McNamara, C. W., Yeung, B. K., Winzeler, E. A., Diagana, T. T., and Kirk, K. (2013) Na(+) regulation in the malaria parasite *Plasmodium falciparum* involves the cation ATPase PfATP4 and is a target of the spiroindolone antimalarials. *Cell Host Microbe 13*, 227−237.
(22) Yeung, B. K., Zou, B., Rottmann, M., Lakshminarayana, S. B., Ang, S. H., Leong, S. Y., Tan, J., Wong, J., Keller-Maerki, S., Fischli, C., Goh, A., Schmitt, E. K., Krastel, P., Francotte, E., Kuhen, K., Plouffe, D., Henson, K., Wagner, T., Winzeler, E. A., Petersen, F., Brun, R., Dartois, V., Diagana, T. T., and Keller, T. H. (2010) Spirotetrahydro β-carbolines (spiroindolones): A new class of potent and orally efficacious compounds for the treatment of malaria. *J. Med. Chem. 53*, 5155−5164.
(23) Tse, M. T. (2010) Antimalarial drugs: Speeding to a new lead. *Nat. Rev. Drug Discovery 9*, 842.
(24) White, N. J., Pukrittayakamee, S., Phyo, A. P., Rueangweerayut, R., Nosten, F., Jittamala, P., Jeeyapant, A., Jain, J. P., Lefevre, G., Li, R., Magnusson, B., Diagana, T. T., and Leong, F. J. (2014) Spiroindolone KAE609 for falciparum and vivax malaria. *N. Engl. J. Med. 371*, 403−410.
(25) Flannery, E. L., Fidock, D. A., and Winzeler, E. A. (2013) Using genetic methods to define the targets of compounds with antimalarial activity. *J. Med. Chem. 56*, 7761−7771.
(26) Hoepfner, D., McNamara, C. W., Lim, C. S., Studer, C., Riedl, R., Aust, T., McCormack, S. L., Plouffe, D. M., Meister, S., Schuierer, S., Plikat, U., Hartmann, N., Staedtler, F., Cotesta, S., Schmitt, E. K., Petersen, F., Supek, F., Glynne, R. J., Tallarico, J. A., Porter, J. A., Fishman, M. C., Bodenreider, C., Diagana, T. T., Movva, N. R., and Winzeler, E. A. (2012) Selective and specific inhibition of the *Plasmodium falciparum* lysyl-tRNA synthetase by the fungal secondary metabolite cladosporin. *Cell Host Microbe 11*, 654−663.
(27) Nam, T. G., McNamara, C. W., Bopp, S., Dharia, N. V., Meister, S., Bonamy, G. M., Plouffe, D. M., Kato, N., McCormack, S., Bursulaya, B., Ke, H., Vaidya, A. B., Schultz, P. G., and Winzeler, E. A. (2011) A chemical genomic analysis of decoquinate, a *Plasmodium falciparum* cytochrome b inhibitor. *ACS Chem. Biol. 6*, 1214−1222.
(28) Bopp, S. E. R., Manary, M. J., Bright, A. T., Johnston, G. L., Dharia, N. V., Luna, F. L., McCormack, S., Plouffe, D., McNamara, C. W., Walker, J. R., Fidock, D. A., Denchi, E. L., and Winzeler, E. A. (2013) Mitotic evolution of *Plasmodium falciparum* shows a stable core genome but recombination in antigen families. *PLoS Genet. 9*, e1003293.
(29) Henderson, P. J., and Lardy, H. A. (1970) Bongkrekic acid. An inhibitor of the adenine nucleotide translocase of mitochondria. *J. Biol. Chem. 245*, 1319−1326.
(30) Nkrumah, L. J., Muhle, R. A., Moura, P. A., Ghosh, P., Hatfull, G. F., Jacobs, W. R., Jr., and Fidock, D. A. (2006) Efficient site-specific integration in *Plasmodium falciparum* chromosomes mediated by mycobacteriophage Bxb1 integrase. *Nat. Methods 3*, 615−621.
(31) Spangenberg, T., Burrows, J. N., Kowalczyk, P., McDonald, S., Wells, T. N., and Willis, P. (2013) The open access malaria box: A drug discovery catalyst for neglected diseases. *PLoS One 8*, e62906.
(32) van Pelt-Koops, J., Pett, H., Graumans, W., van der Vegte-Bolmer, M., van Gemert, G., Rottmann, M., Yeung, B., Diagana, T., and Sauerwein, R. (2012) The spiroindolone drug candidate NITD609 potently inhibits gametocytogenesis and blocks *Plasmodium falciparum* transmission to Anopheles mosquito vector. *Antimicrob. Agents Chemother. 56*, 3544−3548.
(33) Blagborough, A. M., Delves, M. J., Ramakrishnan, C., Lal, K., Butcher, G., and Sinden, R. E. (2013) Assessing transmission blockade in *Plasmodium* spp. *Methods Mol. Biol. 923*, 577−600.
(34) Kirk, K., and Lehane, A. M. (2014) Membrane transport in the malaria parasite and its host erythrocyte. *Biochem. J. 457*, 1−18.
(35) Kirk, K. (2001) Membrane transport in the malaria-infected erythrocyte. *Physiol Rev. 81*, 495−537.
(36) Nagle, A., Wu, T., Kuhen, K., Gagaring, K., Borboa, R., Francek, C., Chen, Z., Plouffe, D., Lin, X., Caldwell, C., Ek, J., Skolnik, S., Liu, F., Wang, J., Chang, J., Li, C., Liu, B., Hollenbeck, T., Tuntland, T., Isbell, J., Chuan, T., Alper, P. B., Fischli, C., Brun, R., Lakshminarayana, S. B., Rottmann, M., Diagana, T. T., Winzeler, E. A., Glynne, R., Tully, D. C., and Chatterjee, A. K. (2012) Imidazolopiperazines: Lead optimization of the second-generation antimalarial agents. *J. Med. Chem. 55*, 4244−4273.
(37) Guiguemde, W. A., Shelat, A. A., Garcia-Bustos, J. F., Diagana, T. T., Gamo, F. J., and Guy, R. K. (2012) Global phenotypic screening for antimalarials. *Chem. Biol. 19*, 116−129.
(38) Fry, M., and Pudney, M. (1992) Site of action of the antimalarial hydroxynaphthoquinone, 2-[trans-4-(4′-chlorophenyl) cyclohexyl]-3-hydroxy-1,4-naphthoquinone (566C80). *Biochem. Pharmacol. 43*, 1545−1553.
(39) Srivastava, I. K., Morrisey, J. M., Darrouzet, E., Daldal, F., and Vaidya, A. B. (1999) Resistance mutations reveal the atovaquone-binding domain of cytochrome b in malaria parasites. *Mol. Microbiol. 33*, 704−711.
(40) Bueno, J. M., Herreros, E., Angulo-Barturen, I., Ferrer, S., Fiandor, J. M., Gamo, F. J., Gargallo-Viola, D., and Derimanov, G. (2012) Exploration of 4(1*H*)-pyridones as a novel family of potent antimalarial inhibitors of the plasmodial cytochrome bc1. *Future Med. Chem. 4*, 2311−2323.
(41) Nilsen, A., LaCrue, A. N., White, K. L., Forquer, I. P., Cross, R. M., Marfurt, J., Mather, M. W., Delves, M. J., Shackleford, D. M., Saenz, F. E., Morrisey, J. M., Steuten, J., Mutka, T., Li, Y., Wirjanata, G., Ryan, E., Duffy, S., Kelly, J. X., Sebayang, B. F., Zeeman, A.-M., Noviyanti, R., Sinden, R. E., Kocken, C. H. M., Price, R. N., Avery, V. M., Angulo-Barturen, I., Jiménez-Díaz, M. B., Ferrer, S., Herreros, E., Sanz, L. M., Gamo, F.-J., Bathurst, I., Burrows, J. N., Siegl, P., Guy, R. K., Winter, R. W., Vaidya, A. B., Charman, S. A., Kyle, D. E., Manetsch, R., and Riscoe, M. K. (2013) Quinolone-3-diarylethers: A new class of antimalarial drug. *Sci. Transl. Med. 5*, 177ra137.
(42) Radloff, P. D., Philipps, J., Nkeyi, M., Hutchinson, D., and Kremsner, P. G. (1996) Atovaquone and proguanil for *Plasmodium falciparum* malaria. *Lancet 347*, 1511−1514.
(43) Perlin, D. S., Seto-Young, D., and Monk, B. C. (1997) The plasma membrane H(+)-ATPase of fungi. A candidate drug target? *Ann. N.Y. Acad. Sci. 834*, 609−617.
(44) Kortagere, S., Welsh, W. J., Morrisey, J. M., Daly, T., Ejigiri, I., Sinnis, P., Vaidya, A. B., and Bergman, L. W. (2010) Structure-based design of novel small-molecule inhibitors of *Plasmodium falciparum*. *J. Chem. Inf. Model. 50*, 840−849.
(45) Douse, C. H., Green, J. L., Salgado, P. S., Simpson, P. J., Thomas, J. C., Langsley, G., Holder, A. A., Tate, E. W., and Cota, E. (2012) Regulation of the *Plasmodium* motor complex: Phosphorylation of myosin A tail-interacting protein (MTIP) loosens its grip on MyoA. *J. Biol. Chem. 287*, 36968−36977.
(46) Green, J. L., Martin, S. R., Fielden, J., Ksagoni, A., Grainger, M., Yim Lim, B. Y., Molloy, J. E., and Holder, A. A. (2006) The MTIP-myosin A complex in blood stage malaria parasites. *J. Mol. Biol. 355*, 933−941.
(47) Goodyer, I. D., and Taraschi, T. F. (1997) *Plasmodium falciparum*: A simple, rapid method for detecting parasite clones in microtiter plates. *Exp. Parasitol. 86*, 158−160.
(48) Manary, M. J., Singhakul, S. S., Flannery, E. L., Bopp, S. E., Corey, V. C., Bright, A. T., McNamara, C. W., Walker, J. R., and Winzeler, E. A. (2014) Identification of pathogen genomic variants through an integrated pipeline. *BMC Bioinf. 15*, 63.
(49) Gregory, J. A., Li, F., Tomosada, L. M., Cox, C. J., Topol, A. B., Vinetz, J. M., and Mayfield, S. (2012) Algae-produced Pfs25 elicits antibodies that inhibit malaria transmission. *PLoS One 7*, e37179.